PHOSPHATE UTILIZATION BY DIATOMS[1]

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Routine measurements of inorganic phosphorus concentration are frequently made on standard oceanographic stations to ascertain the potential productivity of marine waters. In quantitative, ecological studies on diatom populations in the Atlantic Ocean, Riley, Stommel and Bumpus (1949) have utilized the phosphate concentration as a nutrient index. Their equation of productivity depends upon the experimental results of Ketchum (1939), who found that phosphate concentrations below 0.55 microgram-atoms per liter limited the growth of *Nitzschia closterium.*

Riley, Stommel and Bumpus (1949) further checked the validity of this index in their photosynthetic rate equation by calculating the phosphate requirements of a growing plankton population, assuming that 100 carbon atoms to one atom of phosphorus are needed for normal metabolism. However, in studies on the fresh water diatom *Asterionella formosa,* Lund (1950) found that the phosphate content of the plant varied by a factor of seventy in both natural and cultural populations. He stated that the cells are able to store phosphorus in excess of their immediate requirements even at very low levels of phosphate concentration.

The data on the amount of phosphate contained in marine phytoplankton, as found by Ketchum and Redfield (1949), show considerable variation, making the determination of phosphate requirement difficult and uncertain. The use of phosphate as a parameter in the computation of productivity would appear to be premature therefore, until such time as definitive values of the phosphate requirement shall have been established. Even then the total mineral requirements relative to one another must be known and their seasonal and geographic abundances must be shown to vary with biological activity. The use of an arbitrary and easily measured nutrient index for correlation with diatom abundances seems to be unwarranted.

The release of phosphate by marine plankton is an important factor in ecological investigations. Cooper (1935) found that one-fourth to one-half of the phosphate added as phytoplankton in sea water was set free to the environment as inorganic phosphate after one month. Seiwell and Seiwell (1938) reported similar results.

Because of the scarcity of experimental data on the uptake of phosphate and its subsequent release from diatoms, controlled experiments using radioactive phosphate as a tracer were devised to follow the path of phosphate quantitatively from the marine environment to the plant as a function of time. This present study initiates a series of investigations which will attempt to determine by modern radiochemical techniques the minimal quantities of mineral nutrients needed for optimal growth and multiplication of a diatom species in culture.

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Laboratory Techniques

Stock cultures

Single cells or colonies of cells were isolated from inshore water samples taken at the pier of the Scripps Institution of Oceanography, La Jolla, California. Isolated cells of a given species were washed by passing through three drops of sterile nutrient sea water medium, and were then placed in 10 ml. of medium in a test tube. When the diatom population had reached approximately 1000 cells per ml., a one-ml. aliquot was transferred to a cotton-stoppered 250-ml. Erlenmeyer flask containing 100 ml. of a sterile nutrient sea water medium, as described below. Cultures were maintained by transferring one ml. of culture to a flask of fresh medium at two-week intervals. The original cells of the *Asterionella japonica* culture used in the early part of the work had been isolated from the plankton sample in the fall of 1948; the initial cells of the *Asterionella japonica* later utilized were isolated from the plankton samples in the winter of 1950.

Preparation of the nutrient sea water medium

A basic requirement in the radiochemical assay of the partition of phosphate between the nutrient medium and the diatoms is that the total phosphate be contained only within these two fractions. Thus, any phosphate precipitation during the preparation of the enriched sea water or during the course of the experiments invalidates the measurements. Microscopic examination of culture waters prepared in the normal way revealed particulate matter which was found by chemical analysis to be iron phosphate. This solid phase was able to adsorb and to contain a considerable portion of the phosphate. Since the cells often clung to, or were entrapped, or covered by these masses, these precipitates tended to obscure the diatom cells during visual counting, making the counting more difficult. Therefore, the sea water collected for use in the culture medium was subjected to special treatment before and during enrichment to prevent any formation of particulate matter.

The sea water used in the preparation of the medium was collected in glass containers, approximately 14 liters at a time, from the pier. It was filtered through either S & S No. 489 Blue Ribbon or Whatman No. 42 paper before enrichment with nutrients.

The medium used was a modification of Allen and Nelson's (1910) culture solution (Table I). The sea water and the nutrients were sterilized in separate containers. The salts were sterilized in two portions: one, a combination of the KNO_3, $MnCl_2$, and Na_2HAsO_4; the other, the phosphate made up to 100 ml. of

Table I

Culture medium nutrients

Solution		Amount of solution added per liter
M/10	KNO_3	20 ml.
M/100	$MnCl_2$	0.1 ml.
M/100	$CoCl_2$	0.1 ml.
M/100	Na_2HAsO_4	0.1 ml.
3M/1000	K_2HPO_4	Varied through investigation
M/10	Ferric citrate	0.1 ml.

solution with distilled water. The sea water was also sterilized in two portions of three liters each in 4-liter reagent bottles. Sterilization was achieved in the autoclave at 120° C. and 18 pounds pressure. Exposure times were 10 minutes for the salts and 25 minutes for the sea water. After cooling, the water and salt solutions were combined in a sterile 9-liter serum bottle, the salt solution entering through a separatory funnel as the sea water simultaneously passed through glass tubing to the bottom of the bottle. The KNO_3 combination was added with the first three liters of sea water, and the phosphate with the second portion of the sea water. The iron citrate solution was added directly to the culture bottles before diatom inoculation.

Bio-assay

Water samples sometimes failed to produce growth in spite of enrichment. To avoid cultural failures, all of the water collected was first assayed. After preparation of the enriched water, a sample was drawn and placed in several stock culture flasks and then inoculated. An initial count was made and the cultures re-counted twice at weekly intervals. Not infrequently these preliminary assays showed that, in spite of enrichment, the water was refractory to diatom growth. Such water was never used in the radiochemical experiments.

Radiochemical culture techniques

Seven hundred ml. of the enriched sea water was added to the one-liter wide-mouthed culture flasks (Fig. 1), which were stoppered with cotton plugs. The water within the culture bottles was stirred by means of a floating magnetic stirrer made from a 50-ml. round bottom boiling flask. In initial experiments in which the magnetic stirring rod was allowed to rest on the bottom of the culture bottles, the diatoms were destroyed. The solutions were stirred for a period of one minute out of every fifteen minutes with a Haydon Repeat Cycle Timer. It was found that at this stirring rate all of the diatoms received adequate light for normal growth. Continuous stirring resulted in some diatoms clinging to the walls of the glass vessel.

Six such flasks were kept in a constant temperature room at 18° ± 2° C. Five of the flasks contained diatoms while the sixth flask was used as a radiochemical control. The six culture flasks received an equal illumination from a 48-inch, forty-watt fluorescent lamp. The light intensity, as measured with a Weston Photronic Foot Candle Meter, was 55 ± 5 foot candles.

Diatoms were introduced at an inoculation level of about 400 diatoms/ml. Simultaneously, a 10-microcurie aliquot of P^{32} as phosphate ion [2] in sea water was added so that the counting rate of one ml. of the initial solution was about 3000–4000 counts per minute as measured under an end-window Geiger-Muller tube with a window thickness of 1.4 mg./cm.^{2} and a geometry of nine per cent.

Daily radiochemical assays were made on the cultures to determine the phosphate content of the total solution, the diatoms, and the liquid medium freed of diatoms by the following method: one ml. of the whole solution (after continuous stirring for five minutes) was evaporated to dryness under an infrared lamp on a 10

[2] The radioactive P^{32} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

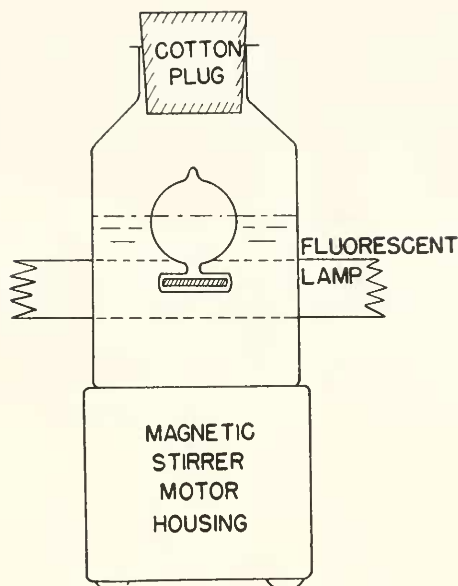

FIGURE 1. The diatom culture bottle. Six such bottles were aligned in front of a single fluorescent lamp for a single experimental run.

cm.[2] copper planchet. When the activity is plotted as a function of time, the known decay rate of 14.3 days of P^{32} is obtained (Fig. 2). This result indicated that there was no loss of phosphate during the experiment by adsorption on the walls of the glass vessel or by the clinging of diatoms to the walls or bottom of the culture flask. It further revealed that, within experimental error, the volume of diatoms at maximum growth was small with respect to the one-ml. aliquot, otherwise a dilution of the activity with time would have been observed.

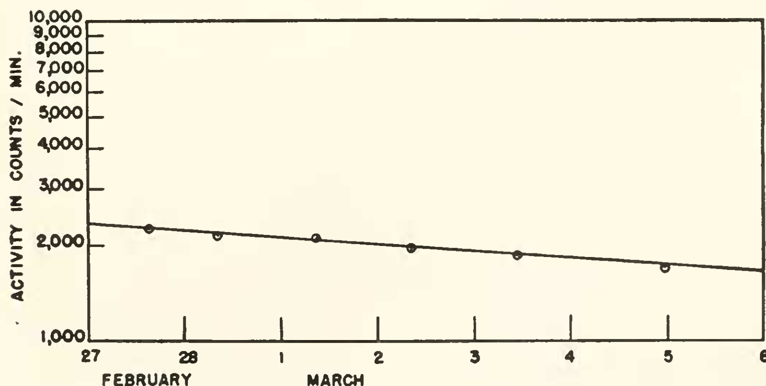

FIGURE 2. The radioactivity of the phosphate in an aliquot of the culture medium containing the diatoms is indicated by the open circles. The theoretical decay of P^{32} is shown by the heavy line. Data from experiment 3T.

Three ml. of the whole solution was centrifuged at 2000 rpm. and one ml. of the supernatant liquid was evaporated to dryness under an infrared lamp on a 10 cm.^{2} copper planchet. The activity of the supernatant liquid, a_s, was assayed under the Geiger-Muller tube and compared to the whole solution, a_t. We thus arrive at the value of phosphate per diatom, P_d, at any given time t, the time of withdrawal of the aliquots from the culture bottles as

$$P_d = \frac{P_t(1 - a_s/a_t)}{N}, \tag{1}$$

where P_t is the initial concentration of phosphate in the culture medium in microgram atoms/ml. and N is the total number of diatoms per ml.

The diatom population assay was made in the following way. One-ml. aliquots were withdrawn from the stirred cultures at the time that the samples for radiochemical analysis were taken. A sufficiently representative population of this

TABLE II

Culture flask	N, cells/ml.		P_s, micro-gram atoms/liter		Growth constant, hours	Final P_d, microgram-atoms $\times 10^8$
	Initial	Final	Initial	Final		
1L	318	122,000	12.8	4.4	15	6.9
2L	612	48,000		4.3	15	8.9
3L	1059	68,000		8.5	17	6.3
4L	450	76,000		5.4	13	9.7
5L	625	68,000		4.5	17	6.6
				Average with std. dev.		7.7 ± 1.5
1O	1760	80,000	8.4	4.2	20	5.3
2O	1970	40,000		4.4	20	10.1
3O	2610	62,000		4.8	20	5.7
4O	1320	54,000		4.0	16	8.2
5O	1140	40,000		6.4	20	4.9
				Average with std. dev.		6.8 ± 2.2
1T	783	84,000	4.2	0.59	13	4.3
2T	327	72,000		0.57	11	5.0
3T	396	75,000		0.69	11	3.3
4T	420	43,000		0.76	12	7.7
5T	321	40,000		1.22	14	7.5
				Average with std. dev.		5.6 ± 2.0
1W	66	52,000	2.7	0.25	17	3.9
2W	253	30,000		0.25	12	5.0
3W	375	41,000		0.20	12	6.0
4W	429	52,000		0.15	12	4.9
5W	267	45,000		0.22	12	5.7
				Average with std. dev.		5.1 ± 0.8

was counted to obtain the density of the population expressed as cells per ml. Care was taken that the cells counted were normal and healthy. Moribund or dead cells were not numerous except in aged cultures, *i.e.,* in some cultures made in early experiments before the bio-assays were made.

The supernatant liquid of the radiochemical control flask was assayed radiochemically, and its activity was compared with that of the whole solution. Any difference would indicate phosphate precipitation. In all of the experiments the activity of the supernatant liquid and the whole solution of the radiochemical control flask were the same within experimental error.

The phosphate analyses were made by the Wooster and Rakestraw (1951) modification of the method of Deniges-Atkins on the solution before the addition of the radioactive phosphate solution. The maximum concentration of phosphate introduced by this addition was 0.01 micro-gram atoms/liter. The phosphate remaining in solution, P_s, at any time t is given by

$$P_s = (a_s/a_t)P_t. \tag{2}$$

Results

Diatom culture experiments were carried out in media varying in initial phosphate levels from 2.7 to 12.8 microgram-atoms per liter. Table II shows the results found between the diatom populations and the phosphate in solution. Columns 2 and 3 represent the initial and final cell count of the diatoms, the final count representing the population when the first signs of leveling-off appeared in the cultures. It should be noted in column 3 that essentially the same final concentrations of diatoms were obtained in all runs. Columns 4 and 5 represent the initial and leveling-off values of the phosphate remaining in solution. The growth constant, or the time for a given cell to divide, is given in column 6. The final concentration of phosphate per diatom as calculated from equation (1) is given in column 7. It is seen that the final phosphate content of the diatoms and the maximum cell population are essentially independent of either the initial inoculum or the initial phosphate level. The spread in the figures is undoubtedly due to cumulative errors in counting of the diatoms, radiochemical assay and sampling techniques.

If one takes as the phosphate content per diatom 5.0×10^{-8} microgram-atoms per cell and a population of 50,000 cells/ml., it is seen that 2.5 microgram-atoms of phosphate per liter are needed for a further division. In experiments T and W there was not enough phosphate remaining in solution to support much further growth.

The dynamic aspects of the phosphate uptake by diatoms are presented in Figures 3 and 4. Figure 3 depicts the relationship between the phosphate remaining in solution and the population level during an experimental run. It is seen that the point at which the diatoms reached their peak growth coincides closely with the leveling-off of the phosphate uptake at 0.25 microgram-atoms per liter. Evident in Table II, in the four replicate culture flasks of run W, is the leveling-off of the phosphate uptake and diatom growth which occurred at phosphate concentrations in the solution between 0.15 and 0.25 micro-gram atoms of phosphate-phosphorus per liter. It was further found that after the leveling-off period, the phos-

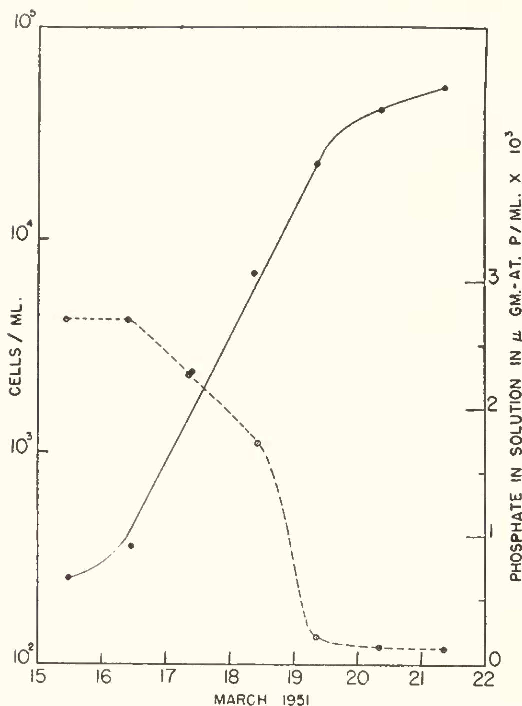

FIGURE 3. Phosphate depletion from solution in an actively growing culture. The heavy curve with solid points represents the exponential growth of diatoms, while the broken curve depicts the phosphate remaining in solution. Data from experiment 2W.

phate content was reduced even further. At this point the number of cells produced by a division is equal to the number that die or become dormant. But since death does not entail the immediate return of phosphate to solution, the available phosphate for uptake is only that which remains in a dissolved state. It was found experimentally that further divisions could take place even below 0.1 microgram atoms of phosphate-phosphorus per liter.

Figure 4 depicts typical relationships between the dissolved phosphate concentration and the phosphate fixed per diatom in a single experimental series. The phosphate content per diatom is observed to vary nearly linearly with the phosphate in the surrounding medium. We may thus write that

$$P_d = A + BP_s, \tag{3}$$

where A and B are constants. A would then be related to the minimal phosphate concentration per diatom, while B would be a function of the initial phosphate concentration for any given experiment. It should be noted that all points plotted in Figure 3 were taken while the diatoms were on the exponential growth curve.

To check the accuracy of the minimal phosphate content per diatom as determined radiochemically, diatoms were grown in the nutrient medium with no added phosphate. The diatoms were assayed daily for population density. At the

Table III

Culture flask	Final cell population, diatoms/ml.	P_d, micro-gram-atoms/diatom $\times 10^8$
1	25,000	4.3
2	22,000	6.6
3	28,000	5.1
4	28,000	3.1
5	25,000	4.9
6	25,000	5.7

approach of the leveling-off period, the diatoms were filtered onto a Whatman No. 42 7-centimeter filter paper disc. The total phosphate content of the diatoms contained in the known volume of the culture flask was determined by digesting the filter paper in 5 ml. of a 2:2:1 nitric: perchloric: sulfuric acid solution until fumes of sulfuric acid appeared. The residue was diluted to 100 ml. and an aliquot assayed for inorganic phosphate. Blank filter paper was simultaneously treated to determine the correction factor due to phosphate contained in the filter paper and the reagents. The corrected results of P_d obtained by this procedure are listed in Table III. It is manifest that they may be compared favorably with the minimal values obtained by the radiochemical method. The initial phosphate concentration was 1.1 microgram atoms of phosphate-phosphorus per liter, indicating that essentially all of the phosphate was utilized in diatom production.

One series of experiments was conducted on phosphate regeneration. The di-

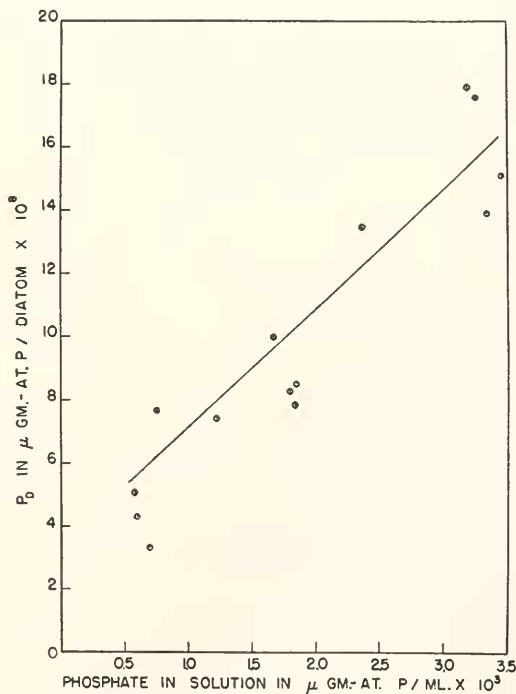

Figure 4. The phosphate fixed per diatom versus the phosphate concentration of the surrounding solution. Data from experimental run T.

atoms after reaching maximum growth were placed in a darkened box at 17° ± 2° C. with no stirring. Daily aliquots were taken and the supernatant liquid analyzed. As is shown in Figure 5, very little of the phosphate returned to solution after three weeks. This experiment was initiated at a low phosphate level. It would thus appear that the minimal phosphate is strongly bound. Although we may treat this experiment as somewhat of a laboratory phenomenon where the breakdown of the diatom is a function of the bacteria present, it nevertheless indicates the stable character of the bound phosphate. Further, in dynamic considerations considerably more time for death and decay of diatoms must be allowed for the return of phosphate to solution.

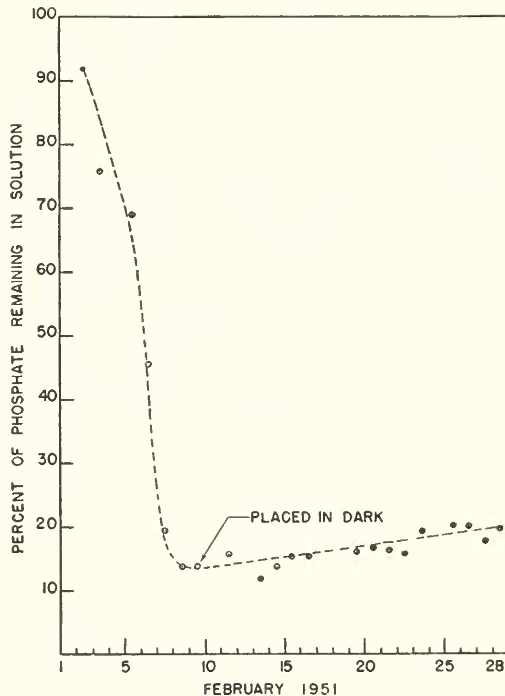

FIGURE 5. The regeneration of phosphate to the solution by diatoms deprived of light. The rapid uptake from February 2 to February 9 represents the diatom growth. Data from experiment 2Q.

In some initial experiments involving diatoms containing more than the minimal amount of phosphate, the phosphate per diatom was determined by collecting a known number of organisms on filter paper, and subsequently assaying radiochemically under the Geiger-Muller tube. However, in this process, it was found that washing the diatoms with radiophosphate-free sea water, to free them of any adhering phosphate in the nutrient medium, removed as much as 50 per cent of the phosphate from the diatoms. Thus a significant fraction of the phosphate above the minimal amount appears to be loosely bound and readily exchangeable, and we may designate it as the labile phosphate.

Discussion

From this dynamic study of the assimilation of phosphate by the marine diatom *Asterionella japonica,* it is evident that these plants have a phosphate content dependent upon that of the environment. This phenomenon, not heretofore fully established, adequately explains the anomalies in phosphate content of diatoms in both cultural and natural populations. Lund's experiments on the fresh water species *Asterionella formosa* first suggested this relationship which he designated as phosphate storage. He found that cells grown in stock culture of 74 microgram-atoms of phosphate-phosphorus per liter contained 3.7×10^{-8} microgram atoms of phosphate-phosphorus per diatom, whereas the cells in stock cultures of only 19 microgram-atoms of phosphate-phosphorus per liter contained but 1.5×10^{-8} microgram-atoms of phosphate-phosphorus each.

By these radiochemical techniques the minimum phosphate content of a diatom can be ascertained, which in the case of the species studied was about 5×10^{-8} microgram-atoms of phosphate-phosphorus per cell. This phosphate may best be designated as "bound phosphate" or the phosphate which is not readily exchangeable with that of the surroundings. Such a condition as this must obtain whenever the phosphate is incorporated irreversibly into the protoplasm. The labile or readily exchangeable phosphate should in no sense be considered as storage since this phosphate level reflects the environmental phosphate through the uptake process.

Inasmuch as this single species was able to flourish in phosphate concentrations lower than the minimum levels found in the euphotic zone of the Pacific Ocean and in surface sea water to which no phosphate had been added, it appears that the inorganic phosphate content of marine waters is representative only of the total potential population that it could support, assuming no diffusion of this nutrient. From the data in Table II of the culture experiments it can be seen that there is a limiting growth factor other than phosphate or nitrate. Moreover, sea water media enriched a second time with all nutrients or with all nutrients but phosphate and nitrate failed to produce growth of diatoms. Using this same technique on other essential elements, the authors hope to establish further minimum mineral requirements of this diatom.

The relationship between phosphate uptake and the concentration of nitrate can be qualitatively seen from the experimental data. The initial nitrate concentration in all experiments was constant at an excess of 2000 microgram-atoms per liter. From Figure 4 we see that initially the phosphate per diatom is about 16×10^{-8} microgram-atoms phosphate-phosphorus per cell, where the phosphate in solution is 3.5 microgram-atoms phosphate-phosphorus/liter. From Table II, experiment 3T, we find that at a final phosphate concentration of 4.2 microgram-atoms phosphate-phosphorus/liter, the cell content is about 5×10^{-8} microgram-atoms of phosphate-phosphorus. Thus, a high nitrate/phosphate ratio is reflected in a high phosphate content of the diatom cells. This result is in agreement with the work of Ketchum (1939).

In the present study it was not considered essential or possible to have bacteria-free diatoms. First of all, only the exponential growth phase of diatom populations was studied. Accordingly, the biomass consisted almost entirely of actively growing plants. There was thus no substrate for bacterial growth. The remarkably close correspondence between the curves of phosphate depletion and

diatom growth would only result if the phosphate was entirely assimilated by the diatoms. The culture medium was autoclaved to remove any other competing living forms.

The diatom stock cultures which were used for inoculation were never freed of bacteria, inasmuch as they were healthy and normal cells. It seemed unlikely that any number of repeated washings could free these cells from bacteria which were attached between the frustule and the cell wall. Observations on decaying diatoms showed that the decay takes place within the frustule and the frustule does not open until the decay process is nearly complete. The standard procedure for determining sterility in diatom cultures is normally carried out on cells which have been repeatedly washed and never on aged cultures. It would seem that the only test for sterility should be one of direct observation as to whether the moribund cells decay.

Summary

1. Studies were made on the uptake of phosphorus by the marine diatom *Asterionella japonica*, utilizing radioactive phosphorus as a tracer.

2. The higher phosphate concentrations in the solution were linearly related to higher contents of phosphate in the diatom cells.

3. A minimal cell concentration of phosphate for further growth was established for this species.

4. Two types of phosphate were found in the cells: the minimal content which was strongly bound and not removed by water washing of the diatoms and a labile phosphate which could be removed. Experiments on the release of phosphate conducted on diatoms containing only minimal amounts of it, indicated that only a small percentage of the phosphate was returned to solution as soluble phosphate after three weeks.

LITERATURE CITED

Allen, E. J., and E. W. Nelson, 1910. On the artificial culture of marine plankton organisms. *J. Mar. Biol. Assoc.*, **8**: 421–474.

Cooper, L. H. N., 1935. The rate of liberation of phosphate in sea water by the breakdown of plankton organisms. *J. Mar. Biol. Assoc.*, **20**: 197–202.

Ketchum, B. H., 1939. The absorption of phosphate and nitrate by illuminated cultures of *Nitzschia closterium*. *Amer. J. Bot.*, **26**: 399–407.

Ketchum, B. H., and A. C. Redfield, 1949. Some physical and chemical characteristics of algae grown in mass culture. *J. Cell. Comp. Physiol.*, **33**: 281–300.

Lund, J. W. G., 1950. Studies on *Asterionella formosa* Haas II. Nutrient depletion and the spring maximum. *J. Ecol.*, **38**: 15–35.

Riley, G. A., H. Stommel and D. F. Bumpus, 1949. Quantitative ecology of the plankton of the western north Atlantic. *Bull. Bingham Oceanogr. Coll.*, **12**: 1–169.

Seiwell, H. R., and G. E. Seiwell, 1938. The sinking of decomposing plankton in sea water and its relationship to oxygen consumption and phosphorous liberation. *Proc. Amer. Philos. Soc.*, **78**: 465–482.

Wooster, W. S., and N. W. Rakestraw, 1951. The estimation of inorganic phosphate in sea water. *J. Mar. Res.*, **10**: 91–100.